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(54) Title: ANTISENSE MODULATION OF APOLIPOPROTEIN(A) EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of apolipoprotein(a). The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding apolipoprotein(a). Methods of using these compounds for modulating of apolipoprotein(a) expression and for treatment of diseases associated with expression of apolipoprotein(a) are provided.

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## ANTISENSE MODULATION OF APOLIPOPROTEIN(A) EXPRESSION

## FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of apolipoprotein(a).

5 In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding apolipoprotein(a). Such compounds have been shown to modulate the expression of apolipoprotein(a).

## 10 BACKGROUND OF THE INVENTION

Lipoproteins are globular, micelle-like particles that consist of a non-polar core of acylglycerols and cholesteryl esters, surrounded by an amphiphilic coating consisting of protein, phospholipid and cholesterol.

15 Lipoproteins have been classified into five broad categories on the basis of their functional and physical properties: chylomicrons (which transport dietary lipids from intestine to tissues), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low  
20 density lipoproteins (LDL), (all of which transport triacylglycerols and cholesterol from the liver to tissues), and high density lipoproteins (HDL) (which transport endogenous cholesterol from tissues to the liver).

25 Lipoprotein particles undergo continuous metabolic processing and have variable properties and compositions. Lipoprotein densities increase without decreasing particle diameter because the density of their outer coatings is less than that of the inner core. The protein components of  
30 lipoproteins are known as apolipoproteins. At least nine apolipoproteins are distributed in significant amounts among the various human lipoproteins.

Lipoprotein(a) (also known as Lp(a)) is a cholesterol

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rich particle of the pro-atherogenic LDL class. Because Lp(a) is found only in Old World primates and European hedgehogs, it has been suggested that it does not play an essential role in lipid and lipoprotein metabolism. Most studies have shown that high concentrations of Lp(a) are strongly associated with increased risk of cardiovascular disease (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). These observations have stimulated numerous studies in humans and other primates to investigate the factors that control Lp(a) concentrations and physiological properties (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61).

Lp(a) contains two disulfide-linked distinct proteins, apolipoprotein(a) (or ApoA) and apolipoprotein B (or ApoB) (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). Apolipoprotein(a) is a unique apolipoprotein encoded by the LPA gene which has been shown to exclusively control the physiological concentrations of Lp(a) (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61). It varies in size due to interallelic differences in the number of tandemly repeated Kringle 4-encoding 5.5 kb sequences in the LPA gene (Rainwater and Kammerer, *J. Exp. Zool.*, 1998, 282, 54-61).

Cloning of human apolipoprotein(a) in 1987 revealed homology to human plasminogen (McLean et al., *Nature*, 1987, 330, 132-137). The gene locus LPA encoding apolipoprotein(a) was localized to chromosome 6q26-27, in close proximity to the homologous gene for plasminogen (Frank et al., *Hum. Genet.*, 1988, 79, 352-356).

Transgenic mice expressing human apolipoprotein(a) were found to be more susceptible than control mice to the development of lipid-staining lesions in the aorta and, consequently, apolipoprotein(a) is co-localized with lipid deposition in the artery walls (Lawn et al., *Nature*, 1992,

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360, 670-672). As an extension of these studies, it was established that the major *in vivo* action of apolipoprotein(a) is inhibition of the conversion of plasminogen to plasmin which causes decreased activation of latent transforming growth factor-beta. Because transforming growth factor-beta is a negative regulator of smooth muscle cell migration and proliferation, inhibition of plasminogen activation indicates a possible mechanism for apolipoprotein(a) induction of atherosclerotic lesions (Grainger et al., *Nature*, 1994, 370, 460-462).

Elevated plasma levels of Lp(a), caused by increased expression of apolipoprotein(a), are associated with increased risk for atherosclerosis and its manifestations, which include hypercholesterolemia (Seed et al., *N. Engl. J. Med.* 1990, 322, 1494-1499), myocardial infarction (Sandkamp et al., *Clin. Chem.*, 1990, 36, 20-23), and thrombosis (Nowak-Gottl et al., *Pediatrics*, 1997, 99, E11).

Moreover, the plasma concentration of Lp(a) is strongly influenced by heritable factors and is refractory to most drug and dietary manipulation (Katan and Beynen, *Am. J. Epidemiol.*, 1987, 125, 387-399; Vessby et al., *Atherosclerosis*, 1982, 44, 61-71). Pharmacologic therapy of elevated Lp(a) levels has been only modestly successful and apheresis remains the most effective therapeutic modality (Hajjar and Nachman, *Annu. Rev. Med.*, 1996, 47, 423-442).

Morishita et al. have reported the use of ribozyme oligonucleotides against apolipoprotein(a) for inhibition of apolipoprotein(a) expression in HepG2 cells (Morishita et al., *Circulation*, 1998, 98, 1898-1904).

Disclosed and claimed in US patent 5,721,138 are nucleotide sequences encoding the human apolipoprotein(a) gene 5'-regulatory region and isolated nucleotide sequences comprising at least thirty consecutive complementary nucleotides from human apolipoprotein(a) from nucleotide

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position -208 to -1448 (Lawn, 1998).

To date, investigative and therapeutic strategies aimed at inhibiting apolipoprotein(a) function have involved the previously cited use of Lp(a) apheresis and ribozyme oligonucleotides. Consequently, there remains a long-felt need for additional agents capable of effectively inhibiting apolipoprotein(a) function.

Antisense technology is emerging as an effective means of reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic and research applications involving modulation of apolipoprotein(a) expression.

The present invention provides compositions and methods for modulating apolipoprotein(a) expression.

#### 15 SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding apolipoprotein(a), and which modulate the expression of apolipoprotein(a).

20 Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of apolipoprotein(a) in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of apolipoprotein(a), by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding

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apolipoprotein(a), ultimately modulating the amount of apolipoprotein(a) produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding apolipoprotein(a).

5 As used herein, the terms "target nucleic acid" and "nucleic acid encoding apolipoprotein(a)" encompass DNA encoding apolipoprotein(a), RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric

10 compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with

15 include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and

20 catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of apolipoprotein(a). In the context of the present invention, "modulation" means either an increase

25 (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for

30 antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a

35 cellular gene (or mRNA transcribed from the gene) whose

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expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding apolipoprotein(a). The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding apolipoprotein(a), regardless of the sequence(s) of such codons.

It is also known in the art that a translation

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termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are

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directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen

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bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the  
5 oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when  
10 binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under  
15 conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

20 Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to  
25 which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

30 Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular  
35 genes. Antisense compounds are also used, for example, to

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distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

For use in kits and diagnostics, the antisense  
5 compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and  
10 tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for  
15 differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the  
20 presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, 2000, 480, 17-24; Celis, et al., *FEBS*  
25 *Lett.*, 2000, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, 2000, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, 1999, 303, 258-72), TOGA (total gene expression analysis)  
30 (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, 2000, 480, 2-16; Jungblut, et al., *Electrophoresis*, 1999, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, 2000, 480, 2-

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16; Larsson, et al., *J. Biotechnol.*, 2000, 80, 143-57),  
subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal.*  
*Biochem.*, 2000, 286, 91-98; Larson, et al., *Cytometry*,  
2000, 41, 203-208), subtractive cloning, differential  
5 display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*,  
2000, 3, 316-21), comparative genomic hybridization  
(Carulli, et al., *J. Cell Biochem. Suppl.*, 1998, 31, 286-  
96), FISH (fluorescent in situ hybridization) techniques  
(Going and Gusterson, *Eur. J. Cancer*, 1999, 35, 1895-904)  
10 and mass spectrometry methods (reviewed in (To, *Comb. Chem.*  
*High Throughput Screen*, 2000, 3, 235-41).

The specificity and sensitivity of antisense is also  
harnessed by those of skill in the art for therapeutic  
uses. Antisense oligonucleotides have been employed as  
15 therapeutic moieties in the treatment of disease states in  
animals and man. Antisense oligonucleotide drugs,  
including ribozymes, have been safely and effectively  
administered to humans and numerous clinical trials are  
presently underway. It is thus established that  
20 oligonucleotides can be useful therapeutic modalities that  
can be configured to be useful in treatment regimes for  
treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term  
"oligonucleotide" refers to an oligomer or polymer of  
25 ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or  
mimetics thereof. This term includes oligonucleotides  
composed of naturally-occurring nucleobases, sugars and  
covalent internucleoside (backbone) linkages as well as  
oligonucleotides having non-naturally-occurring portions  
30 which function similarly. Such modified or substituted  
oligonucleotides are often preferred over native forms  
because of desirable properties such as, for example,  
enhanced cellular uptake, enhanced affinity for nucleic

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acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends  
5 other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50  
10 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic  
15 RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes  
20 of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be  
25 linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to  
30 form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and  
35 DNA is a 3' to 5' phosphodiester linkage.

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Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;

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5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;  
5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;  
5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555;  
5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of  
5 which are commonly owned with this application, and each of  
which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do  
not include a phosphorus atom therein have backbones that  
are formed by short chain alkyl or cycloalkyl  
10 internucleoside linkages, mixed heteroatom and alkyl or  
cycloalkyl internucleoside linkages, or one or more short  
chain heteroatomic or heterocyclic internucleoside  
linkages. These include those having morpholino linkages  
(formed in part from the sugar portion of a nucleoside);  
15 siloxane backbones; sulfide, sulfoxide and sulfone  
backbones; formacetyl and thioformacetyl backbones;  
methylene formacetyl and thioformacetyl backbones;  
riboacetyl backbones; alkene containing backbones;  
sulfamate backbones; methyleneimino and methylenehydrazino  
20 backbones; sulfonate and sulfonamide backbones; amide  
backbones; and others having mixed N, O, S and CH<sub>2</sub> component  
parts.

Representative United States patents that teach the  
preparation of the above oligonucleosides include, but are  
25 not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444;  
5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564;  
5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677;  
5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;  
5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;  
30 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and  
5,677,439, certain of which are commonly owned with this  
application, and each of which is herein incorporated by  
reference.

In other preferred oligonucleotide mimetics, both the  
35 sugar and the internucleoside linkage, i.e., the backbone,

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of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has  
5 been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are  
10 retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of  
15 which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and  
20 oligonucleosides with heteroatom backbones, and in particular  $-CH_2-NH-O-CH_2-$ ,  $-CH_2-N(CH_3)-O-CH_2-$  [known as a methylene (methylimino) or MMI backbone],  $-CH_2-O-N(CH_3)-CH_2-$ ,  $-CH_2-N(CH_3)-N(CH_3)-CH_2-$  and  $-O-N(CH_3)-CH_2-CH_2-$  [wherein the native phosphodiester backbone is represented as  $-O-P-O-CH_2-$   
25 ] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

30 Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl,  
35 alkenyl and alkynyl may be substituted or unsubstituted  $C_1$

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to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to about 10. Other preferred oligonucleotides

5 comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino,

10 polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar

15 properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as

20 described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>, also described in examples hereinbelow.-

25 A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>-)<sub>n</sub> group bridging the 2' oxygen

30 atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F).

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The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ( $-C\equiv C-CH_3$ ) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-

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trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and

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are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen

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sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion.

- 5 Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol
- 10 moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem.*
- 15 *Let.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al.,
- 20 *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a
- 25 polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-
- 30 carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen,

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(S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethacin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds.

"Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more

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chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

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The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis.

Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

10 The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

15 The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.:

5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;  
25 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;  
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;  
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;  
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;  
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of  
30 which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable  
35 of providing (directly or indirectly) the biologically

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active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such  
5 prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals  
10 and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphatel] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S.  
15 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do  
20 not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the  
25 like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66,  
30 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and  
35 isolating the free acid in the conventional manner. The

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free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as

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ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and  
5 include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not  
10 limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric  
15 acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic  
20 acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

25 The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the  
30 expression of apolipoprotein(a) is treated by administering antisense compounds in accordance with this invention. The  
~~compounds of the invention can be utilized in~~  
pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically  
35 acceptable diluent or carrier. Use of the antisense

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compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful  
5 for research and diagnostics, because these compounds hybridize to nucleic acids encoding apolipoprotein(a), enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid  
10 encoding apolipoprotein(a) can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of  
15 apolipoprotein(a) in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered  
20 in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or  
25 insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or  
30 infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for  
35 topical administration may include transdermal patches,

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ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants.

10 Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. 15 dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be 20 complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, 25 dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical 30 formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, 35 nanoparticulates, suspensions or solutions in water or non-

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aqueous media, capsules, gel capsules, sachets, tablets or minitabets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which

- 5 oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include
- 10 chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,
- 15 Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an
- 20 acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred
- 25 combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles,
- 30 or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include
- ~~poly-amino-acids; polyimines; polyacrylates;~~
- polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates,
- 35 polyethyleneglycols (PEG) and starches;

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polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, 5 protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcyanoacrylate), DEAE-methacrylate, DEAE- 10 hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in 15 detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

20 Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and 25 other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but 30 are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional 35 techniques well known in the pharmaceutical industry. Such

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techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the  
5 active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as,  
10 but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances  
15 which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as  
20 foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such  
25 compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

#### Emulsions

30 The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in *Pharmaceutical Dosage Forms*,  
35 Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker,

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Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o

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emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*,

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Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, 5 lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers 10 especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium 15 aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, 20 fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage* 25 *Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, 30 carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form

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colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

5           Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations  
10 include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical  
15 scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

20           The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,  
25 N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988,  
30 Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are

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among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical*

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*Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

10 Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol  
15 monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprates (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain  
20 alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared  
25 without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives  
30 of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides,  
35 saturated polyglycolized C8-C10 glycerides, vegetable oils

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and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w  
5 and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of  
10 improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms,  
15 improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be  
20 particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion  
25 compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids  
30 within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration

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enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

#### Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages in vivo.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

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Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper

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epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable  
5 complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res.*  
10 *Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs.  
15 Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal*  
20 *of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine  
25 (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed  
30 from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of  
35 liposomes containing interferon to guinea pig skin resulted

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in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study  
5 tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

10 Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl  
15 dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems  
20 were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to  
25 liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming  
30 lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art

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that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside  $G_{M1}$ , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside  $G_{M1}$  or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that

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liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

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Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly  
5 deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their  
10 targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of  
15 serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes.  
20 The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the  
25 most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is  
30 classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic  
35 esters such as ethylene glycol esters, propylene glycol

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esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block  
5 polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant  
10 is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and  
15 sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant  
20 is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry  
25 either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations  
30 and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

#### Penetration Enhancers

In one embodiment, the present invention employs  
35 various penetration enhancers to effect the efficient

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delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic

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- acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-
- 5 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C<sub>1-10</sub> alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in*
- 10 *Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

Bile salts: The physiological role of bile includes

15 the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic

20 derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable

25 sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate),

30 taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ~~ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-~~

~~fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE)~~ (Lee et al., *Critical*

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Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto et al., J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita et al., J. Pharm. Sci., 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This

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class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

#### Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other

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extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be  
5 reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

#### 10 Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to  
15 an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical  
20 pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium  
25 sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium  
30 acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.)...

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which

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do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, 5 alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous 10 solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for 15 non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, 20 magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components 25 conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or 30 anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, 35 such materials, when added, should not unduly interfere

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with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J.

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When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and

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repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on  $EC_{50}$ s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

#### EXAMPLES

##### Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis  
25 Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was

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increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods (Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

#### 2'-Fluoro amidites

##### 2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously (Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841) and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a  $S_N2$ -displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

##### 2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and

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triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

5        2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard  
10 procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by  
15 selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are  
20 prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially  
25 available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner.  
30 After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca.

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400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 5 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a 10 white solid, mp 222-4°C).

**2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel 15 and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 20 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading 25 onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was 30 co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 35 M) was added and the reaction stirred for an additional one

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hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with  $\text{CH}_3\text{CN}$  (200 mL). The residue was dissolved in  $\text{CHCl}_3$  (1.5 L) and extracted with 2x500 mL of saturated  $\text{NaHCO}_3$  and 2x500 mL of saturated  $\text{NaCl}$ . The organic phase was dried over  $\text{Na}_2\text{SO}_4$ , filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with  $\text{EtOAc}$ /hexane/acetone (5:5:1) containing 0.5%  $\text{Et}_3\text{NH}$ . The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

15      3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M),  $\text{DMF}$ /pyridine (750 mL of a 3:1 mixture prepared from 562 mL of  $\text{DMF}$  and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of  $\text{MeOH}$ . Upon completion of the reaction, as judged by TLC,  $\text{MeOH}$  (50 mL) was added and the mixture evaporated at  $35^\circ\text{C}$ . The residue was dissolved in  $\text{CHCl}_3$  (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated  $\text{NaCl}$ . The water layers were back extracted with 200 mL of  $\text{CHCl}_3$ . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using  $\text{EtOAc}$ /hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

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3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over

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sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

5        2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was  
10 evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica  
15 column using EtOAc/hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

20        N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting  
25 mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and the extracts were  
30 combined, dried over MgSO<sub>4</sub> and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting  
solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

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2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites (also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (R<sub>f</sub> 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

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5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxo)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-

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methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over  $P_2O_5$  under high vacuum for two days at 40°C. The  
5 reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red  
10 coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a  
15 flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-  
20 formadoximinooxy)ethyl]-5-methyluridine  
2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry  $CH_2Cl_2$  (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was  
25 filtered, the filtrate was washed with ice cold  $CH_2Cl_2$ , and the combined organic phase was washed with water, brine and dried over anhydrous  $Na_2SO_4$ . The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20%  
30 aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine as white foam (1.95 g, 78%).

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5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-  
dimethylaminoxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-  
formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol)  
5 was dissolved in a solution of 1M pyridinium p-  
toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium  
cyanoborohydride (0.39g, 6.13mmol) was added to this  
solution at 10°C under inert atmosphere. The reaction  
mixture was stirred for 10 minutes at 10°C. After that the  
10 reaction vessel was removed from the ice bath and stirred  
at room temperature for 2 h, the reaction monitored by TLC  
(5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was  
added and extracted with ethyl acetate (2x20mL). Ethyl  
acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to  
15 dryness. Residue was dissolved in a solution of 1M PPTS in  
MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was  
added and the reaction mixture was stirred at room  
temperature for 10 minutes. Reaction mixture cooled to 10°C  
in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol)  
20 was added and reaction mixture stirred at 10°C for 10  
minutes. After 10 minutes, the reaction mixture was  
removed from the ice bath and stirred at room temperature  
for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL)  
solution was added and extracted with ethyl acetate  
25 (2x25mL). Ethyl acetate layer was dried over anhydrous  
Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was  
purified by flash column chromatography and eluted with 5%  
MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-  
[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam  
30 (14.6g, 80%).

2'-O-(dimethylaminoxyethyl)-5-methyluridine

~~Triethylamine trihydrofluoride~~ (3.91mL, 24.0mmol) was  
dissolved in dry THF and triethylamine (1.67mL, 12mmol,  
dry, kept over KOH). This mixture of triethylamine-2HF was

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then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine 2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was

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dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-  
5 2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites (also known in  
10 the art as 2'-O-(aminooxyethyl) nucleoside amidites) are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-  
15 ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside  
20 may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine  
25 deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-  
30 dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may

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phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalamidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

5 2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are  
10 prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl  
uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50  
15 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O<sup>2</sup>-, 2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed,  
20 placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous  
25 layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent.  
30 As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

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5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x200 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers are washed with saturated NaHCO<sub>3</sub> solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-

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benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and  
5 deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by  
10 reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are  
15 prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

20 Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are  
25 prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

30 Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

### Example 3

#### Oligonucleoside Synthesis

35 Methylenemethylimino linked oligonucleosides, also

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identified as MMI linked oligonucleosides, methylenedi-methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked  
5 oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023,  
10 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

15 Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

#### Example 4

##### PNA Synthesis

20 Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance  
25 with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

#### Example 5

##### Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed  
30 oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap"

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segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric  
Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo-  
10 nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-  
15 methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support  
20 and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The  
25 pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then  
30 analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

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[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

5 [2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

10 [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above  
15 procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization  
20 utilizing 3, H-1, 2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides  
25 are synthesized according to United States patent 5,623,065, herein incorporated by reference.

#### Example 6

##### Oligonucleotide Isolation

After cleavage from the controlled pore glass column  
30 (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by

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polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by <sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* 1991, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

#### Example 7

##### Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH<sub>4</sub>OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

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**Example 8****Oligonucleotide Analysis - 96 Well Plate Format**

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

**Example 9****Cell culture and oligonucleotide treatment**

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 7 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

**T-24 cells:**

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented

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with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely  
5 passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be  
10 seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained  
15 from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per  
20 mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

25 Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up  
30 to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from  
the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium  
35 (Clonetics Corporation, Walkersville MD) formulated as

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recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

HepG2 cells:

5       The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were routinely cultured in Eagle's MEM supplemented with 10% fetal calf serum, non-essential amino acids, and 1 mM sodium pyruvate (Gibco/Life Technologies, 10 Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

15       For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

AML12 cells:

20       AML12 (alpha mouse liver 12) cell line was established from hepatocytes from a mouse (CD1 strain, line MT42) transgenic for human TGF alpha. Cells are cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 25 ng/ml selenium, and 40 ng/ml dexamethasone, and 90%; 10% fetal bovine serum. For subculturing, spent medium is removed and fresh media of 0.25% trypsin, 0.03% EDTA solution is added. Fresh trypsin solution (1 to 2 ml) is added and the culture is left to sit at room temperature 30 until the cells detach.

Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

35       For Northern blotting or other analyses, cells may be

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seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Primary mouse hepatocytes:

- 5        Primary mouse hepatocytes were prepared from CD-1 mice purchased from Charles River Labs (Wilmington, MA) and were routinely cultured in Hepatocyte Attachment Media (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies, Gaithersburg, MD), 250nM dexamethasone  
10       (Sigma), and 10nM bovine insulin (Sigma). Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 10000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells are plated onto 100 mm or other standard tissue culture plates  
15       coated with rat tail collagen (200ug/mL) (Becton Dickinson) and treated similarly using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

- When cells reach 80% confluency, they are treated with  
20       oligonucleotide. For cells grown in 96-well plates, wells are washed once with 200  $\mu$ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130  $\mu$ L of OPTI-MEM™-1 containing 3.75  $\mu$ g/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7  
25       hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal  
30       oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, TCCGTCATCGCTCCTCAGGG, SEQ ID NO: 1, a 2'-O-methoxyethyl  
35       gapmer (2'-O-methoxyethyls shown in bold) with a

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phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with  
5 a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new  
10 oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in  
15 subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

#### Example 10

#### 20 Analysis of oligonucleotide inhibition of apolipoprotein(a) expression

Antisense modulation of apolipoprotein(a) expression can be assayed in a variety of ways known in the art. For example, apolipoprotein(a) mRNA levels can be quantitated  
25 by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example,  
30 Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume

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1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of apolipoprotein(a) can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to apolipoprotein(a) can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

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**Example 11****Poly(A)+ mRNA isolation**

Poly(A)+ mRNA can be isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium is removed from the cells and each well is washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) is added to each well, the plate is gently agitated and then incubated at room temperature for five minutes. 55  $\mu$ L of lysate is transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates are incubated for 60 minutes at room temperature, washed 3 times with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate is blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C is added to each well, the plate is incubated on a 90°C hot plate for 5 minutes, and the eluate is then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

**Example 12****Total RNA Isolation**

Total RNA can be isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium is removed from the cells and each well is washed with 200  $\mu$ L cold

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PBS. 100  $\mu$ L Buffer RLT is added to each well and the plate vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol is then added to each well and the contents mixed by pipetting three times up and down. The samples are then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum is applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE is then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum is applied for an additional 10 minutes. The plate is then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate is then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA is then eluted by pipetting 60  $\mu$ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step is repeated with an additional 60  $\mu$ L water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

#### Example 13

#### Real-time Quantitative PCR Analysis of apolipoprotein(a) mRNA Levels

Quantitation of apolipoprotein(a) mRNA levels can be determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput

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quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are

5 quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon

10 Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe.

15 When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-

20 exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye

25 molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from

30 untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated

35 for their ability to be "multiplexed" with a GAPDH

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amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each  
5 dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both  
10 the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for  
15 that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents are obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions are carried out by adding 25  $\mu$ L PCR cocktail (1x TAQMAN<sup>™</sup> buffer A, 5.5 mM  
20 MgCl<sub>2</sub>, 300  $\mu$ M each of dATP, dCTP and dGTP, 600  $\mu$ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD<sup>™</sup>, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25  $\mu$ L total RNA solution. The RT reaction is  
25 carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD<sup>™</sup>, 40 cycles of a two-step PCR protocol are carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

30 Gene target quantities obtained by real time RT-PCR can be normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen<sup>™</sup> (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real  
35 time RT-PCR, by being run simultaneously with the target,

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5 multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes (Eugene, OR). Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, 1998, 265, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a  
10 CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human apolipoprotein(a) are designed to hybridize to a human apolipoprotein(a) sequence, using published sequence information (GenBank  
15 accession number NM\_005577, incorporated herein as SEQ ID NO: 3).

For human GAPDH the standard PCR primers are: forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 4) reverse primer: GAAGATGGTGATGGGATTTTC (SEQ ID NO: 5) and the PCR probe is:  
20 5' JOE-CAAGCTTCCC GTTCTCAGCC-TAMRA 3' (SEQ ID NO: 6) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

#### Example 14

#### 25 Northern blot analysis of apolipoprotein(a) mRNA levels

Eighteen hours after antisense treatment, cell monolayers are washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA is prepared following manufacturer's recommended protocols.

30 Twenty micrograms of total RNA is fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a-MOPS buffer system (AMRESCO, Inc., Solon, OH). RNA is transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway,  
35 NJ) by overnight capillary transfer using a

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Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer is confirmed by UV visualization. Membranes are fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, 5 La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human apolipoprotein(a), a human apolipoprotein(a) specific probe is prepared by PCR using a 10 forward and a reverse primer. To normalize for variations in loading and transfer efficiency, membranes are stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes are visualized and quantitated 15 using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data are normalized to GAPDH levels in untreated controls.

#### Example 15

Chimeric phosphorothioate oligonucleotides having 2'-MOE 20 wings and a deoxy gap targeting human apolipoprotein(a)

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human apolipoprotein(a) RNA, using published sequence (GenBank accession number NM\_005577, incorporated 25 herein as SEQ ID NO: 3). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in 30 length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate 35 (P=S) throughout the oligonucleotide. All cytidine

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residues are 5-methylcytidines.

Table 1

Chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap targeting human apolipoprotein(a)

5	ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	SEQ ID NO
	144367	Coding	3	174	GGCAGGTCCTTCCTGTGACA	7
	144368	Coding	3	352	TCTGCGTCTGAGCATTGCGT	8
	144369	Coding	3	522	AAGCTTGGCAGGTTCTTCCT	9
	144370	Coding	3	1743	TCGGAGGCGCGACGGCAGTC	10
10	144371	Coding	3	2768	CGGAGGCGCGACGGCAGTCC	11
	144372	Coding	3	2910	GGCAGGTTCTTCCTGTGACA	12
	144373	Coding	3	3371	ATAACAATAAGGAGCTGCCA	13
	144374	Coding	3	4972	GACCAAGCTTGGCAGGTTCT	14
	144375	Coding	3	5080	TAACAATAAGGAGCTGCCAC	15
15	144376	Coding	3	5315	TGACCAAGCTTGGCAGGTTT	16
	144377	Coding	3	5825	TTCTGCGTCTGAGCATTGCG	17
	144378	Coding	3	6447	AACAATAAGGAGCTGCCACA	18
	144379	Coding	3	7155	ACCTGACACCGGGATCCCTC	19
	144380	Coding	3	7185	CTGAGCATTGCGTCAGGTTG	20
20	144381	Coding	3	8463	AGTAGTTCATGATCAAGCCA	21
	144382	Coding	3	8915	GACGGCAGTCCCTTCTGCGT	22
	144383	Coding	3	9066	GGCAGGTTCTTCCAGTGACA	23
	144384	Coding	3	10787	TGACCAAGCTTGGCAAGTTC	24
	144385	Coding	3	11238	TATAACACCAAGGACTAATC	25
25	144386	Coding	3	11261	CCATCTGACATTGGGATCCA	26
	144387	Coding	3	11461	TGTGGTGTTCATAGAGGACCA	27
	144388	Coding	3	11823	ATGGGATCCTCCGATGCCAA	28
	144389	Coding	3	11894	ACACCAAGGGCGAATCTCAG	29
	144390	Coding	3	11957	TTCTGTCACTGGACATCGTG	30
30	144391	Coding	3	12255	CACACGGATCGGTTGTGTAA	31
	144392	Coding	3	12461	ACATGTCCTTCCTGTGACAG	32
	144393	Coding	3	12699	CAGAAGGAGGCCCTAGGCTT	33
	144394	Coding	3	13354	CTGGCGGTGACCATGTAGTC	34
	144395	3'UTR	3	13711	TCTAAGTAGGTTGATGCTTC	35
35	144396	3'UTR	3	13731	TCCTTACCCACGTTTCAGCT	36
	144397	3'UTR	3	13780	GGAACAGTGTCTTCGTTTGA	37
	144398	3'UTR	3	13801	GTTTGGCATAGCTGGTAGCT	38
	144399	3'UTR	3	13841	ACCTTAAAAGCTTATACACA	39
	144400	3'UTR	3	13861	ATACAGAATTTGTCTAGTCAG	40
40	144401	3'UTR	3	13881	GTCATAGCTATGACACCTTA	41

#### Example 16

Western blot analysis of apolipoprotein(a) protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h

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after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to apolipoprotein(a) is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

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What is claimed is:

1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding human apolipoprotein(a), wherein said compound specifically hybridizes with a nucleic acid molecule encoding human apolipoprotein(a) and inhibits the expression of human apolipoprotein(a).
2. The compound of claim 1 which is an antisense oligonucleotide.
3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or 41.
4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.
6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.
7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.
8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.
9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.
10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.
11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding human apolipoprotein(a).

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12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of human apolipoprotein(a) in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of human apolipoprotein(a) is inhibited.

16. A method of treating a human having a disease or condition associated with human apolipoprotein(a) comprising administering to said human a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of human apolipoprotein(a) is inhibited.

17. The method of claim 16 wherein the condition involves abnormal lipid metabolism.

18. The method of claim 16 wherein the condition involves abnormal cholesterol metabolism.

19. The method of claim 16 wherein the condition is atherosclerosis.

20. The method of claim 16 wherein the disease is cardiovascular disease.

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## SEQUENCE LISTING

<110> Isis Pharmaceuticals, Inc.  
 Rosanne M. Crooke  
 Mark J. Graham

<120> ANTISENSE MODULATION OF APOLIPOPROTEIN(A) EXPRESSION

<130> ISPH-0690

<150> 09/923,515

<151> 2001-08-07

<160> 41

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<220>

<221> CDS

<222> (46)...(13692)

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 Glu Val Val Leu Leu Leu Leu Phe Leu Lys Ser Ala Ala Pro Glu  
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caa agc cat gtg gtc cag gat tgc tac cat ggt gat gga cag agt tat	153
Gln Ser His Val Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr	
25 30 35	
cga ggc acg tac tcc acc act gtc aca gga agg acc tgc caa gct tgg	201
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp	
40 45 50	
tca tct atg aca cca cat caa cat aat agg acc aca gaa aac tac cca	249
Ser Ser Met Thr Pro His Gln His Asn Arg Thr Thr Glu Asn Tyr Pro	
55 60 65	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca	297
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala	
70 75 80	
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc	345
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys	
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aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg	393
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro	
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act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg	441
Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
120 125 130	
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	489
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
135 140 145	
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	537
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
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gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	585
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
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Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
185 190 195	
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	681
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
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Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
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cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	777
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-3-

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265 270 275	
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Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro	
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Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro	
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aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg	1257
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Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
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Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
425 430 435	
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg	1401
Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly	
440 445 450	
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct	1449
Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala	
455 460 465	
cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc	1497

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Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln	Glu	Cys		
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gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	gtt	acc	ccg	gtt	cca	agc	cta	1785	
Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	Thr	Pro	Val	Pro	Ser	Leu		
565					570					575					580		
gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	caa	agg	cct	ggg	gtg	cag	1833	
Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln		
				585					590					595			
gag	tgc	tac	cat	ggt	aat	gga	cag	agt	tat	cga	ggc	aca	tac	tcc	acc	1881	
Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr	Arg	Gly	Thr	Tyr	Ser	Thr		
			600					605					610				
act	gtc	aca	gga	aga	acc	tgc	caa	gct	tgg	tca	tct	atg	aca	cca	cac	1929	
Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser	Met	Thr	Pro	His		
		615					620					625					
tgc	cat	agt	cgg	acc	cca	gaa	tac	tac	cca	aat	gct	ggc	ttg	atc	atg	1977	
Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala	Gly	Leu	Ile	Met		
		630				635					640						
aac	tac	tgc	agg	aat	cca	gat	gct	gtg	gca	gct	cct	tat	tgt	tat	acg	2025	
Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro	Tyr	Cys	Tyr	Thr		
645					650					655					660		
agg	gat	ccc	ggt	gtc	agg	tgg	gag	tac	tgc	aac	ctg	acg	caa	tgc	tca	2073	
Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Gln	Cys	Ser		
				665					670					675			
gac	gca	gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	gtt	acc	ccg	gtt	cca	2121	
Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	Thr	Pro	Val	Pro		
			680					685					690				
agc	cta	gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	caa	agg	cct	ggg	2169	
Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly		

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695					700					705						
gtg	cag	gag	tgc	tac	cat	ggt	aat	gga	cag	agt	tat	cga	ggc	aca	tac	2217
Val	Gln	Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr	Arg	Gly	Thr	Tyr	
710					715					720						
tcc	acc	act	gtc	aca	gga	aga	acc	tgc	caa	gct	tgg	tca	tct	atg	aca	2265
Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser	Met	Thr	
725					730					735					740	
cca	cac	tcg	cat	agt	cgg	acc	cca	gaa	tac	tac	cca	aat	gct	ggc	ttg	2313
Pro	His	Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala	Gly	Leu	
745					750					755						
atc	atg	aac	tac	tgc	agg	aat	cca	gat	gct	gtg	gca	gct	cct	tat	tgt	2361
Ile	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro	Tyr	Cys	
760					765					770						
tat	acg	agg	gat	ccc	ggt	gtc	agg	tgg	gag	tac	tgc	aac	ctg	acg	caa	2409
Tyr	Thr	Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Gln	
775					780					785						
tgc	tca	gac	gca	gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	gtt	acc	ccg	2457
Cys	Ser	Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	Thr	Pro	
790					795					800						
gtt	cca	agc	cta	gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	caa	agg	2505
Val	Pro	Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	
805					810					815					820	
cct	ggg	gtg	cag	gag	tgc	tac	cat	ggt	aat	gga	cag	agt	tat	cga	ggc	2553
Pro	Gly	Val	Gln	Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr	Arg	Gly	
825					830					835						
aca	tac	tcc	acc	act	gtc	aca	gga	aga	acc	tgc	caa	gct	tgg	tca	tct	2601
Thr	Tyr	Ser	Thr	Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser	
840					845					850						
atg	aca	cca	cac	tcg	cat	agt	cgg	acc	cca	gaa	tac	tac	cca	aat	gct	2649
Met	Thr	Pro	His	Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala	
855					860					865						
ggc	ttg	atc	atg	aac	tac	tgc	agg	aat	cca	gat	gct	gtg	gca	gct	cct	2697
Gly	Leu	Ile	Met	Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro	
870					875					880						
tat	tgt	tat	acg	agg	gat	ccc	ggt	gtc	agg	tgg	gag	tac	tgc	aac	ctg	2745
Tyr	Cys	Tyr	Thr	Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	
885					890					895					900	
acg	caa	tgc	tca	gac	gca	gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	gtt	2793
Thr	Gln	Cys	Ser	Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	
905					910					915						
acc	ccg	gtt	cca	agc	cta	gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	2841
Thr	Pro	Val	Pro	Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	
920					925					930						

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caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat	2889
Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr	
935 940 945	
cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg	2937
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp	
950 955 960	
tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca	2985
Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro	
965 970 975 980	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca	3033
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala	
985 990 995	
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc	3081
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys	
1000 1005 1010	
aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg	3129
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro	
1015 1020 1025	
act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg	3177
Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
1030 1035 1040	
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	3225
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
1045 1050 1055 1060	
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	3273
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
1065 1070 1075	
gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	3321
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
1080 1085 1090	
tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct	3369
Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
1095 1100 1105	
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	3417
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
1110 1115 1120	
tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg	3465
Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
1125 1130 1135 1140	
cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	3513
Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln	
1145 1150 1155	

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gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn 1160 1165 1170	3561
gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr 1175 1180 1185	3609
tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro 1190 1195 1200	3657
gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro 1205 1210 1215 1220	3705
gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg 1225 1230 1235	3753
tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala 1240 1245 1250	3801
gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser 1255 1260 1265	3849
gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His 1270 1275 1280	3897
ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly 1285 1290 1295 1300	3945
aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg 1305 1310 1315	3993
acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg 1320 1325 1330	4041
aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly 1335 1340 1345	4089
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly 1350 1355 1360	4137
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala 1365 1370 1375 1380	4185
cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc	4233

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Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln	Glu	Cys		
				1385					1390					1395			
tac	cat	ggt	aat	gga	cag	agt	tat	cga	ggc	aca	tac	tcc	acc	act	gtc	4281	
Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr	Arg	Gly	Thr	Tyr	Ser	Thr	Thr	Val		
			1400					1405					1410				
aca	gga	aga	acc	tgc	caa	gct	tgg	tca	tct	atg	aca	cca	cac	tgc	cat	4329	
Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser	Met	Thr	Pro	His	Ser	His		
			1415				1420					1425					
agt	cgg	acc	cca	gaa	tac	tac	cca	aat	gct	ggc	ttg	atc	atg	aac	tac	4377	
Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala	Gly	Leu	Ile	Met	Asn	Tyr		
			1430				1435				1440						
tgc	agg	aat	cca	gat	gct	gtg	gca	gct	cct	tat	tgt	tat	acg	agg	gat	4425	
Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro	Tyr	Cys	Tyr	Thr	Arg	Asp		
			1445			1450				1455					1460		
ccc	ggt	gtc	agg	tgg	gag	tac	tgc	aac	ctg	acg	caa	tgc	tca	gac	gca	4473	
Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Gln	Cys	Ser	Asp	Ala		
				1465					1470					1475			
gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	ggt	acc	ccg	ggt	cca	agc	cta	4521	
Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	Thr	Pro	Val	Pro	Ser	Leu		
			1480					1485					1490				
gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	caa	agg	cct	ggg	gtg	cag	4569	
Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly	Val	Gln		
			1495				1500					1505					
gag	tgc	tac	cat	ggt	aat	gga	cag	agt	tat	cga	ggc	aca	tac	tcc	acc	4617	
Glu	Cys	Tyr	His	Gly	Asn	Gly	Gln	Ser	Tyr	Arg	Gly	Thr	Tyr	Ser	Thr		
			1510			1515					1520						
act	gtc	aca	gga	aga	acc	tgc	caa	gct	tgg	tca	tct	atg	aca	cca	cac	4665	
Thr	Val	Thr	Gly	Arg	Thr	Cys	Gln	Ala	Trp	Ser	Ser	Met	Thr	Pro	His		
			1525			1530				1535					1540		
tgc	cat	agt	cgg	acc	cca	gaa	tac	tac	cca	aat	gct	ggc	ttg	atc	atg	4713	
Ser	His	Ser	Arg	Thr	Pro	Glu	Tyr	Tyr	Pro	Asn	Ala	Gly	Leu	Ile	Met		
				1545					1550					1555			
aac	tac	tgc	agg	aat	cca	gat	gct	gtg	gca	gct	cct	tat	tgt	tat	acg	4761	
Asn	Tyr	Cys	Arg	Asn	Pro	Asp	Ala	Val	Ala	Ala	Pro	Tyr	Cys	Tyr	Thr		
			1560				1565						1570				
agg	gat	ccc	ggt	gtc	agg	tgg	gag	tac	tgc	aac	ctg	acg	caa	tgc	tca	4809	
Arg	Asp	Pro	Gly	Val	Arg	Trp	Glu	Tyr	Cys	Asn	Leu	Thr	Gln	Cys	Ser		
			1575				1580					1585					
gac	gca	gaa	ggg	act	gcc	gtc	gcg	cct	ccg	act	ggt	acc	ccg	ggt	cca	4857	
Asp	Ala	Glu	Gly	Thr	Ala	Val	Ala	Pro	Pro	Thr	Val	Thr	Pro	Val	Pro		
			1590			1595					1600						
agc	cta	gag	gct	cct	tcc	gaa	caa	gca	ccg	act	gag	caa	agg	cct	ggg	4905	
Ser	Leu	Glu	Ala	Pro	Ser	Glu	Gln	Ala	Pro	Thr	Glu	Gln	Arg	Pro	Gly		

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1605	1610	1615	1620	
gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac				4953
Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr	1625	1630	1635	
tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca				5001
Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr	1640	1645	1650	
cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg				5049
Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu	1655	1660	1665	
atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt				5097
Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys	1670	1675	1680	
tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa				5145
Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln	1685	1690	1695	1700
tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg				5193
Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro	1705	1710	1715	
gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag caa agg				5241
Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg	1720	1725	1730	
cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc				5289
Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly	1735	1740	1745	
aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct				5337
Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser	1750	1755	1760	
atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct				5385
Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala	1765	1770	1775	1780
ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct				5433
Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro	1785	1790	1795	
tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg				5481
Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu	1800	1805	1810	
acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt				5529
Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val	1815	1820	1825	
acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag				5577
Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu	1830	1835	1840	

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caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat	5625
Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr	
1845 1850 1855 1860	
cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg	5673
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp	
1865 1870 1875	
tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca	5721
Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro	
1880 1885 1890	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca	5769
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala	
1895 1900 1905	
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc	5817
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys	
1910 1915 1920	
aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg	5865
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro	
1925 1930 1935 1940	
act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg	5913
Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
1945 1950 1955	
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	5961
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
1960 1965 1970	
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	6009
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
1975 1980 1985	
gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	6057
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
1990 1995 2000	
tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct	6105
Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
2005 2010 2015 2020	
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	6153
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
2025 2030 2035	
tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg	6201
Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
2040 2045 2050	
cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	6249
Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln	
2055 2060 2065	

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gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat	6297
Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn	
2070 2075 2080	
gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc	6345
Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr	
2085 2090 2095 2100	
tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca	6393
Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro	
2105 2110 2115	
gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca	6441
Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro	
2120 2125 2130	
gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg	6489
Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg	
2135 2140 2145	
tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc	6537
Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala	
2150 2155 2160	
gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc	6585
Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser	
2165 2170 2175 2180	
gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc tac cat	6633
Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His	
2185 2190 2195	
ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc aca gga	6681
Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly	
2200 2205 2210	
aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg	6729
Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg	
2215 2220 2225	
acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg	6777
Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
2230 2235 2240	
aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt	6825
Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
2245 2250 2255 2260	
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg	6873
Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly	
2265 2270 2275	
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct	6921
Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala	
2280 2285 2290	
cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag gag tgc	6969

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Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys	
2295 2300 2305	
tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc	7017
Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val	
2310 2315 2320	
aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat	7065
Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His	
2325 2330 2335 2340	
agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac	7113
Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr	
2345 2350 2355	
tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat	7161
Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp	
2360 2365 2370	
ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca	7209
Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala	
2375 2380 2385	
gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta	7257
Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu	
2390 2395 2400	
gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg gtg cag	7305
Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln	
2405 2410 2415 2420	
gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac tcc acc	7353
Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr	
2425 2430 2435	
act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca cca cac	7401
Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His	
2440 2445 2450	
tcg cat agt cgg acc cca gaa tac tac cca aat gct ggc ttg atc atg	7449
Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met	
2455 2460 2465	
aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt tat acg	7497
Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr	
2470 2475 2480	
agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa tgc tca	7545
Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser	
2485 2490 2495 2500	
gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg gtt cca	7593
Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro	
2505 2510 2515	
agc cta gag gct cct tcc gaa caa gca ccg act gag caa agg cct ggg	7641
Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly	

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2520	2525	2530	
gtg cag gag tgc tac cat ggt aat gga cag agt tat cga ggc aca tac Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr 2535 2540 2545			7689
tcc acc act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr 2550 2555 2560			7737
cca cac tcg cat agt cgg acc cca gaa tac tac oca aat gct ggc ttg Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu 2565 2570 2575 2580			7785
atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct tat tgt Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys 2585 2590 2595			7833
tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg acg caa Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln 2600 2605 2610			7881
tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt acc ccg Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val Thr Pro 2615 2620 2625			7929
gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag cag agg Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu Gln Arg 2630 2635 2640			7977
cct ggg gtg cag gag tgc tac cac ggt aat gga cag agt tat cga ggc Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Arg Gly 2645 2650 2655 2660			8025
aca tac tcc acc act gtc act gga aga acc tgc caa gct tgg tca tct Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser 2665 2670 2675			8073
atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca aat gct Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro Asn Ala 2680 2685 2690			8121
ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca gct cct Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala Ala Pro 2695 2700 2705			8169
tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc aac ctg Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys Asn Leu 2710 2715 2720			8217
acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg act gtt Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro Thr Val 2725 2730 2735 2740			8265
acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg act gag Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro Thr Glu 2745 2750 2755			8313

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caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag agt tat	8361
Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr	
2760 2765 2770	
cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa gct tgg	8409
Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp	
2775 2780 2785	
tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac tac cca	8457
Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr Tyr Pro	
2790 2795 2800	
aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct gtg gca	8505
Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala Val Ala	
2805 2810 2815 2820	
gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag tac tgc	8553
Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu Tyr Cys	
2825 2830 2835	
aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg cct ccg	8601
Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala Pro Pro	
2840 2845 2850	
act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa gca ccg	8649
Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln Ala Pro	
2855 2860 2865	
act gag caa agg cct ggg gtg cag gag tgc tac cat ggt aat gga cag	8697
Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn Gly Gln	
2870 2875 2880	
agt tat cga ggc aca tac tcc acc act gtc aca gga aga acc tgc caa	8745
Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln	
2885 2890 2895 2900	
gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca gaa tac	8793
Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro Glu Tyr	
2905 2910 2915	
tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca gat gct	8841
Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro Asp Ala	
2920 2925 2930	
gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg tgg gag	8889
Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg Trp Glu	
2935 2940 2945	
tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc gtc gcg	8937
Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala Val Ala	
2950 2955 2960	
cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc gaa caa	8985
Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser Glu Gln	
2965 2970 2975 2980	

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gca ccg act gag cag agg cct ggg gtg cag gag tgc tac cac ggt aat	9033
Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His Gly Asn	
2985 2990 2995	
gga cag agt tat cga ggc aca tac tcc acc act gtc act gga aga acc	9081
Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr	
3000 3005 3010	
tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg acc cca	9129
Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg Thr Pro	
3015 3020 3025	
gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg aat cca	9177
Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg Asn Pro	
3030 3035 3040	
gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt gtc agg	9225
Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly Val Arg	
3045 3050 3055 3060	
tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg act gcc	9273
Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly Thr Ala	
3065 3070 3075	
gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct cct tcc	9321
Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala Pro Ser	
3080 3085 3090	
gaa caa gca ccg act gag cag agg cct ggg gtg cag gag tgc tac cac	9369
Glu Gln Ala Pro Thr Glu Gln Arg Pro Gly Val Gln Glu Cys Tyr His	
3095 3100 3105	
ggt aat gga cag agt tat cga ggc aca tac tcc acc act gtc act gga	9417
Gly Asn Gly Gln Ser Tyr Arg Gly Thr Tyr Ser Thr Thr Val Thr Gly	
3110 3115 3120	
aga acc tgc caa gct tgg tca tct atg aca cca cac tcg cat agt cgg	9465
Arg Thr Cys Gln Ala Trp Ser Ser Met Thr Pro His Ser His Ser Arg	
3125 3130 3135 3140	
acc cca gaa tac tac cca aat gct ggc ttg atc atg aac tac tgc agg	9513
Thr Pro Glu Tyr Tyr Pro Asn Ala Gly Leu Ile Met Asn Tyr Cys Arg	
3145 3150 3155	
aat cca gat gct gtg gca gct cct tat tgt tat acg agg gat ccc ggt	9561
Asn Pro Asp Ala Val Ala Ala Pro Tyr Cys Tyr Thr Arg Asp Pro Gly	
3160 3165 3170	
gtc agg tgg gag tac tgc aac ctg acg caa tgc tca gac gca gaa ggg	9609
Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Ser Asp Ala Glu Gly	
3175 3180 3185	
act gcc gtc gcg cct ccg act gtt acc ccg gtt cca agc cta gag gct	9657
Thr Ala Val Ala Pro Pro Thr Val Thr Pro Val Pro Ser Leu Glu Ala	
3190 3195 3200	
cct tcc gaa caa gca ccg act gag cag agg cct ggg gtg cag gag tgc	9705

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Pro Ser Glu Gln Ala	Pro Thr Glu Gln Arg	Pro Gly Val Gln Glu Cys	
3205	3210	3215	3220
tac cac ggt aat gga	cag agt tat cga	ggc aca tac tcc	acc act gtc 9753
Tyr His Gly Asn Gly	Gln Ser Tyr Arg	Gly Thr Tyr Ser Thr	Thr Val 3235
	3225	3230	
act gga aga acc tgc	caa gct tgg tca	tct atg aca cca	cac tcg cat 9801
Thr Gly Arg Thr Cys	Gln Ala Trp Ser	Met Thr Pro His	Ser His 3250
	3240	3245	
agt cgg acc cca gaa	tac tac cca aat	gct ggc ttg atc	atg aac tac 9849
Ser Arg Thr Pro Glu	Tyr Tyr Pro Asn	Ala Gly Leu Ile	Met Asn Tyr 3265
	3255	3260	
tgc agg aat cca gat	gct gtg gca gct	cct tat tgt tat	acg agg gat 9897
Cys Arg Asn Pro Asp	Ala Val Ala Ala	Pro Tyr Cys Tyr	Thr Arg Asp 3280
	3270	3275	
ccc ggt gtc agg tgg	gag tac tgc aac	ctg acg caa tgc	tca gac gca 9945
Pro Gly Val Arg Trp	Glu Tyr Cys Asn	Leu Thr Gln Cys	Ser Asp Ala 3300
	3285	3290	
gaa ggg act gcc gtc	gcg cct ccg act	gtt acc ccg gtt	cca agc cta 9993
Glu Gly Thr Ala Val	Ala Pro Pro Thr	Val Thr Pro Val	Pro Ser Leu 3315
	3305	3310	
gag gct cct tcc gaa	caa gca ccg act	gag cag agg cct	ggg gtg cag 10041
Glu Ala Pro Ser Glu	Gln Ala Pro Thr	Glu Gln Arg Pro	Gly Val Gln 3330
	3320	3325	
gag tgc tac cac ggt	aat gga cag agt	tat cga ggc aca	tac tcc acc 10089
Glu Cys Tyr His Gly	Asn Gly Gln Ser	Tyr Arg Gly Thr	Tyr Ser Thr 3345
	3335	3340	
act gtc act gga aga	acc tgc caa gct	tgg tca tct atg	aca cca cac 10137
Thr Val Thr Gly Arg	Thr Cys Gln Ala	Trp Ser Ser Met	Thr Pro His 3360
	3350	3355	
tcg cat agt cgg acc	cca gaa tac tac	cca aat gct ggc	ttg atc atg 10185
Ser His Ser Arg Thr	Pro Glu Tyr Tyr	Pro Asn Ala Gly	Leu Ile Met 3380
	3365	3370	
aac tac tgc agg aat	cca gat cct gtg	gca gcc cct tat	tgt tat acg 10233
Asn Tyr Cys Arg Asn	Pro Asp Pro Val	Ala Ala Pro Tyr	Cys Tyr Thr 3395
	3385	3390	
agg gat ccc agt gtc	agg tgg gag tac	tgc aac ctg aca	caa tgc tca 10281
Arg Asp Pro Ser Val	Arg Trp Glu Tyr	Cys Asn Leu Thr	Gln Cys Ser 3410
	3400	3405	
gac gca gaa ggg act	gcc gtc gcg cct	cca act att acc	ccg att cca 10329
Asp Ala Glu Gly Thr	Ala Val Ala Pro	Pro Thr Ile Thr	Pro Ile Pro 3425
	3415	3420	
agc cta gag gct cct	tct gaa caa gca	cca act gag caa	agg cct ggg 10377
Ser Leu Glu Ala Pro	Ser Glu Gln Ala	Pro Thr Glu Gln	Arg Pro Gly

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3430	3435	3440	
gtg cag gag tgc tac cac gga aat gga cag agt tat caa ggc aca tac			10425
Val Gln Glu Cys Tyr His Gly Asn Gly Gln Ser Tyr Gln Gly Thr Tyr			
3445	3450	3455	3460
ttc att act gtc aca gga aga acc tgc caa gct tgg tca tct atg aca			10473
Phe Ile Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser Met Thr			
3465	3470	3475	
cca cac tcg cat agt cgg acc cca gca tac tac cca aat gct ggc ttg			10521
Pro His Ser His Ser Arg Thr Pro Ala Tyr Tyr Pro Asn Ala Gly Leu			
3480	3485	3490	
atc aag aac tac tgc cga aat cca gat cct gtg gca gcc cct tgg tgt			10569
Ile Lys Asn Tyr Cys Arg Asn Pro Asp Pro Val Ala Ala Pro Trp Cys			
3495	3500	3505	
tat aca aca gat ccc agt gtc agg tgg gag tac tgc aac ctg aca cga			10617
Tyr Thr Thr Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Arg			
3510	3515	3520	
tgc tca gat gca gaa tgg act gcc ttc gtc cct ccg aat gtt att ctg			10665
Cys Ser Asp Ala Glu Trp Thr Ala Phe Val Pro Pro Asn Val Ile Leu			
3525	3530	3535	3540
gct cca agc cta gag gct ttt ttt gaa caa gca ctg act gag gaa acc			10713
Ala Pro Ser Leu Glu Ala Phe Phe Glu Gln Ala Leu Thr Glu Glu Thr			
3545	3550	3555	
ccc ggg gta cag gac tgc tac tac cat tat gga cag agt tac cga ggc			10761
Pro Gly Val Gln Asp Cys Tyr Tyr His Tyr Gly Gln Ser Tyr Arg Gly			
3560	3565	3570	
aca tac tcc acc act gtc aca gga aga act tgc caa gct tgg tca tct			10809
Thr Tyr Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ala Trp Ser Ser			
3575	3580	3585	
atg aca cca cac cag cat agt cgg acc cca gaa aac tac cca aat gct			10857
Met Thr Pro His Gln His Ser Arg Thr Pro Glu Asn Tyr Pro Asn Ala			
3590	3595	3600	
ggc ctg acc agg aac tac tgc agg aat cca gat gct gag att cgc cct			10905
Gly Leu Thr Arg Asn Tyr Cys Arg Asn Pro Asp Ala Glu Ile Arg Pro			
3605	3610	3615	3620
tgg tgt tac acc atg gat ccc agt gtc agg tgg gag tac tgc aac ctg			10953
Trp Cys Tyr Thr Met Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu			
3625	3630	3635	
aca caa tgc ctg gtg aca gaa tca agt gtc ctt gca act ctc acg gtg			11001
Thr Gln Cys Leu Val Thr Glu Ser Ser Val Leu Ala Thr Leu Thr Val			
3640	3645	3650	
gtc cca gat cca agc aca gag gct tct tct gaa gaa gca cca acg gag			11049
Val Pro Asp Pro Ser Thr Glu Ala Ser Ser Glu Glu Ala Pro Thr Glu			
3655	3660	3665	

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caa agc ccc ggg gtc cag gat tgc tac cat ggt gat gga cag agt tat Gln Ser Pro Gly Val Gln Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr 3670 3675 3680	11097
cga ggc tca ttc tct acc act gtc aca gga agg aca tgt cag tct tgg Arg Gly Ser Phe Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ser Trp 3685 3690 3695 3700	11145
tcc tct atg aca cca cac tgg cat cag agg aca aca gaa tat tat cca Ser Ser Met Thr Pro His Trp His Gln Arg Thr Thr Glu Tyr Tyr Pro 3705 3710 3715	11193
aat ggt ggc ctg acc agg aac tac tgc agg aat cca gat gct gag att Asn Gly Gly Leu Thr Arg Asn Tyr Cys Arg Asn Pro Asp Ala Glu Ile 3720 3725 3730	11241
agt cct tgg tgt tat acc atg gat ccc aat gtc aga tgg gag tac tgc Ser Pro Trp Cys Tyr Thr Met Asp Pro Asn Val Arg Trp Glu Tyr Cys 3735 3740 3745	11289
aac ctg aca caa tgt cca gtg aca gaa tca agt gtc ctt gcg acg tcc Asn Leu Thr Gln Cys Pro Val Thr Glu Ser Ser Val Leu Ala Thr Ser 3750 3755 3760	11337
acg gct gtt tct gaa caa gca cca acg gag caa agc ccc aca gtc cag Thr Ala Val Ser Glu Gln Ala Pro Thr Glu Gln Ser Pro Thr Val Gln 3765 3770 3775 3780	11385
gac tgc tac cat ggt gat gga cag agt tat cga ggc tca ttc tcc acc Asp Cys Tyr His Gly Asp Gly Gln Ser Tyr Arg Gly Ser Phe Ser Thr 3785 3790 3795	11433
act gtt aca gga agg aca tgt cag tct tgg tcc tct atg aca cca cac Thr Val Thr Gly Arg Thr Cys Gln Ser Trp Ser Ser Met Thr Pro His 3800 3805 3810	11481
tgg cat cag aga acc aca gaa tac tac cca aat ggt ggc ctg acc agg Trp His Gln Arg Thr Thr Glu Tyr Tyr Pro Asn Gly Gly Leu Thr Arg 3815 3820 3825	11529
aac tac tgc agg aat cca gat gct gag att cgc cct tgg tgt tat acc Asn Tyr Cys Arg Asn Pro Asp Ala Glu Ile Arg Pro Trp Cys Tyr Thr 3830 3835 3840	11577
atg gat ccc agt gtc aga tgg gag tac tgc aac ctg acg caa tgt cca Met Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Gln Cys Pro 3845 3850 3855 3860	11625
gtg atg gaa tca act ctc ctc aca act ccc acg gtg gtc cca gtt cca Val Met Glu Ser Thr Leu Leu Thr Thr Pro Thr Val Val Pro Val Pro 3865 3870 3875	11673
agc aca gag ctt cct tct gaa gaa gca cca act gaa aac agc act ggg Ser Thr Glu Leu Pro Ser Glu Glu Ala Pro Thr Glu Asn Ser Thr Gly 3880 3885 3890	11721

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gtc cag gac tgc tac cga ggt gat gga cag agt tat cga ggc aca ctc Val Gln Asp Cys Tyr Arg Gly Asp Gly Gln Ser Tyr Arg Gly Thr Leu 3895 3900 3905	11769
tcc acc act atc aca gga aga aca tgt cag tct tgg tgc tct atg aca Ser Thr Thr Ile Thr Gly Arg Thr Cys Gln Ser Trp Ser Ser Met Thr 3910 3915 3920	11817
cca cat tgg cat cgg agg atc cca tta tac tat cca aat gct ggc ctg Pro His Trp His Arg Arg Ile Pro Leu Tyr Tyr Pro Asn Ala Gly Leu 3925 3930 3935 3940	11865
acc agg aac tac tgc agg aat cca gat gct gag att cgc cct tgg tgt Thr Arg Asn Tyr Cys Arg Asn Pro Asp Ala Glu Ile Arg Pro Trp Cys 3945 3950 3955	11913
tac acc atg gat ccc agt gtc agg tgg gag tac tgc aac ctg aca cga Tyr Thr Met Asp Pro Ser Val Arg Trp Glu Tyr Cys Asn Leu Thr Arg 3960 3965 3970	11961
tgt cca gtg aca gaa tgc agt gtc ctc aca act ccc aca gtg gcc ccg Cys Pro Val Thr Glu Ser Ser Val Leu Thr Thr Pro Thr Val Ala Pro 3975 3980 3985	12009
gtt cca agc aca gag gct cct tct gaa caa gca cca cct gag aaa agc Val Pro Ser Thr Glu Ala Pro Ser Glu Gln Ala Pro Pro Glu Lys Ser 3990 3995 4000	12057
cct gtg gtc cag gat tgc tac cat ggt gat gga cgg agt tat cga ggc Pro Val Val Gln Asp Cys Tyr His Gly Asp Gly Arg Ser Tyr Arg Gly 4005 4010 4015 4020	12105
ata tcc tcc acc act gtc aca gga agg acc tgt caa tct tgg tca tct Ile Ser Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ser Trp Ser Ser 4025 4030 4035	12153
atg ata cca cac tgg cat cag agg acc cca gaa aac tac cca aat gct Met Ile Pro His Trp His Gln Arg Thr Pro Glu Asn Tyr Pro Asn Ala 4040 4045 4050	12201
ggc ctg acc gag aac tac tgc agg aat cca gat tct ggg aaa caa ccc Gly Leu Thr Glu Asn Tyr Cys Arg Asn Pro Asp Ser Gly Lys Gln Pro 4055 4060 4065	12249
tgg tgt tac aca acc gat ccg tgt gtg agg tgg gag tac tgc aat ctg Trp Cys Tyr Thr Thr Asp Pro Cys Val Arg Trp Glu Tyr Cys Asn Leu 4070 4075 4080	12297
aca caa tgc tca gaa aca gaa tca ggt gtc cta gag act ccc act gtt Thr Gln Cys Ser Glu Thr Glu Ser Gly Val Leu Glu Thr Pro Thr Val 4085 4090 4095 4100	12345
gtt cca gtt cca agc atg gag gct cat tct gaa gca gca cca act gag Val Pro Val Pro Ser Met Glu Ala His Ser Glu Ala Ala Pro Thr Glu 4105 4110 4115	12393
caa acc cct gtg gtc cgg cag tgc tac cat ggt aat ggc cag agt tat	12441

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Gln Thr Pro Val Val Arg Gln Cys Tyr His Gly Asn Gly Gln Ser Tyr	
4120 4125 4130	
cga ggc aca ttc tcc acc act gtc aca gga agg aca tgt caa tct tgg	12489
Arg Gly Thr Phe Ser Thr Thr Val Thr Gly Arg Thr Cys Gln Ser Trp	
4135 4140 4145	
tca tcc atg aca cca cac cgg cat cag agg acc cca gaa aac tac cca	12537
Ser Ser Met Thr Pro His Arg His Gln Arg Thr Pro Glu Asn Tyr Pro	
4150 4155 4160	
aat gat ggc ctg aca atg aac tac tgc agg aat cca gat gcc gat aca	12585
Asn Asp Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala Asp Thr	
4165 4170 4175 4180	
ggc cct tgg tgt ttt acc atg gac ccc agc atc agg tgg gag tac tgc	12633
Gly Pro Trp Cys Phe Thr Met Asp Pro Ser Ile Arg Trp Glu Tyr Cys	
4185 4190 4195	
aac ctg acg cga tgc tca gac aca gaa ggg act gtg gtc gct cct ccg	12681
Asn Leu Thr Arg Cys Ser Asp Thr Glu Gly Thr Val Val Ala Pro Pro	
4200 4205 4210	
act gtc atc cag gtt cca agc cta ggg cct cct tct gaa caa gac tgt	12729
Thr Val Ile Gln Val Pro Ser Leu Gly Pro Pro Ser Glu Gln Asp Cys	
4215 4220 4225	
atg ttt ggg aat ggg aaa gga tac cgg ggc aag aag gca acc act gtt	12777
Met Phe Gly Asn Gly Lys Gly Tyr Arg Gly Lys Lys Ala Thr Thr Val	
4230 4235 4240	
act ggg acg cca tgc cag gaa tgg gct gcc cag gag ccc cat aga cac	12825
Thr Gly Thr Pro Cys Gln Glu Trp Ala Ala Gln Glu Pro His Arg His	
4245 4250 4255 4260	
agc acg ttc att cca ggg aca aat aaa tgg gca ggt ctg gaa aaa aat	12873
Ser Thr Phe Ile Pro Gly Thr Asn Lys Trp Ala Gly Leu Glu Lys Asn	
4265 4270 4275	
tac tgc cgt aac cct gat ggt gac atc aat ggt ccc tgg tgc tac aca	12921
Tyr Cys Arg Asn Pro Asp Gly Asp Ile Asn Gly Pro Trp Cys Tyr Thr	
4280 4285 4290	
atg aat cca aga aaa ctt ttt gac tac tgt gat atc cct ctc tgt gca	12969
Met Asn Pro Arg Lys Leu Phe Asp Tyr Cys Asp Ile Pro Leu Cys Ala	
4295 4300 4305	
tcc tct tca ttt gat tgt ggg aag cct caa gtg gag ccg aag aaa tgt	13017
Ser Ser Ser Phe Asp Cys Gly Lys Pro Gln Val Glu Pro Lys Lys Cys	
4310 4315 4320	
cct gga agc att gta ggg ggg tgt gtg gcc cac cca cat tcc tgg ccc	13065
Pro Gly Ser Ile Val Gly Cys Val Ala His Pro His Ser Trp Pro	
4325 4330 4335 4340	
tgg caa gtc agt ctc aga aca agg ttt gga aag cac ttc tgt gga ggc	13113
Trp Gln Val Ser Leu Arg Thr Arg Phe Gly Lys His Phe Cys Gly Gly	

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4345	4350	4355	
acc tta ata tcc cca gag tgg gtg ctg act gct gct cac tgc ttg aag			13161
Thr Leu Ile Ser Pro Glu Trp Val Leu Thr Ala Ala His Cys Leu Lys			
4360	4365	4370	
aag tcc tca agg cct tca tcc tac aag gtc atc ctg ggt gca cac caa			13209
Lys Ser Ser Arg Pro Ser Ser Tyr Lys Val Ile Leu Gly Ala His Gln			
4375	4380	4385	
gaa gtg aac ctc gaa tct cat gtt cag gaa ata gaa gtg tct agg ctg			13257
Glu Val Asn Leu Glu Ser His Val Gln Glu Ile Glu Val Ser Arg Leu			
4390	4395	4400	
ttc ttg gag ccc aca caa gca gat att gcc ttg cta aag cta agc agg			13305
Phe Leu Glu Pro Thr Gln Ala Asp Ile Ala Leu Leu Lys Leu Ser Arg			
4405	4410	4415	4420
cct gcc gtc atc act gac aaa gta atg cca gct tgt ctg cca tcc cca			13353
Pro Ala Val Ile Thr Asp Lys Val Met Pro Ala Cys Leu Pro Ser Pro			
4425	4430	4435	
gac tac atg gtc acc gcc agg act gaa tgt tac atc act ggc tgg gga			13401
Asp Tyr Met Val Thr Ala Arg Thr Glu Cys Tyr Ile Thr Gly Trp Gly			
4440	4445	4450	
gaa acc caa ggt acc ttt ggg act ggc ctt ctc aag gaa gcc cag ctc			13449
Glu Thr Gln Gly Thr Phe Gly Thr Gly Leu Leu Lys Glu Ala Gln Leu			
4455	4460	4465	
ctt gtt att gag aat gaa gtg tgc aat cac tat aag tat att tgt gct			13497
Leu Val Ile Glu Asn Glu Val Cys Asn His Tyr Lys Tyr Ile Cys Ala			
4470	4475	4480	
gag cat ttg gcc aga ggc act gac agt tgc cag ggt gac agt gga ggg			13545
Glu His Leu Ala Arg Gly Thr Asp Ser Cys Gln Gly Asp Ser Gly Gly			
4485	4490	4495	4500
cct ctg gtt tgc ttc gag aag gac aaa tac att tta caa gga gtc act			13593
Pro Leu Val Cys Phe Glu Lys Asp Lys Tyr Ile Leu Gln Gly Val Thr			
4505	4510	4515	
tct tgg ggt ctt ggc tgt gca cgc ccc aat aag cct ggt gtc tat gct			13641
Ser Trp Gly Leu Gly Cys Ala Arg Pro Asn Lys Pro Gly Val Tyr Ala			
4520	4525	4530	
cgt gtt tca agg ttt gtt act tgg att gag gga atg atg aga aat aat			13689
Arg Val Ser Arg Phe Val Thr Trp Ile Glu Gly Met Met Arg Asn Asn			
4535	4540	4545	
taa ttggacggga gacagagtga agcatcaacc tacttagaag ctgaaacgtg			13742
ggtaaggatt tagcatgctg gaaataatag acagcaatca aacgaagaca ctgttcccag			13802
ctaccagcta tgccaaacct tggcattttt ggtatttttg tgtataagct tttaaggctt			13862
gactgacaaa ttctgtatta aggtgtcata gctatgacat ttgttaaaaa taaactctgc			13922
acttattttg atttga			13938

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